

Genome Editing of Isogenic Human Induced Pluripotent Stem Cells Recapitulates Long QT Phenotype for Drug Testing



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ABSTRACT

BACKGROUND Human induced pluripotent stem cells (iPSCs) play an important role in disease modeling and drug testing. However, the current methods are time-consuming and lack an isogenic control.

OBJECTIVES This study sought to establish an efficient technology to generate human PSC-based disease models with isogenic control.

METHODS The ion channel genes *KCNQ1* and *KCNH2* with dominant negative mutations causing long QT syndrome types 1 and 2, respectively, were stably integrated into a safe harbor *AAVS1* locus using zinc finger nuclease technology.

RESULTS Patch-clamp recording revealed that the edited iPSC-derived cardiomyocytes (iPSC-CMs) displayed characteristic long QT syndrome phenotype and significant prolongation of the action potential duration compared with the unedited control cells. Finally, addition of nifedipine (L-type calcium channel blocker) or pinacidil (KATP-channel opener) shortened the action potential duration of iPSC-CMs, confirming the validity of isogenic iPSC lines for drug testing in the future.

CONCLUSIONS Our study demonstrates that iPSC-CM-based disease models can be rapidly generated by over-expression of dominant negative gene mutants. (J Am Coll Cardiol 2014;64:451-9) © 2014 by the American College of Cardiology Foundation.

Predictive disease models, such as animal models and cell lines, play an important role in studying the pathophysiological mechanisms of human disease and developing targeted therapies. However, many human phenotypes fail to be successfully recapitulated in these models because of species differences or a lack of synteny. Human

pluripotent stem cell (PSC) biology has opened a new avenue for disease modeling. There are 2 main types of human PSCs: embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) (1-4). Human PSCs can self-renew indefinitely and can be differentiated to any cell types present in the human body. Because of their properties and origin, human PSCs

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ABBREVIATIONS AND ACRONYMS

APD = action potential duration

CM = cardiomyocyte

ESC = embryonic stem cell

iPSC = induced pluripotent stem cell

LQTS = long QT syndrome

PSC = human pluripotent stem cell

ZFN = zinc finger nuclease

are considered to be an inexhaustible, scalable, and biologically matched material for cardiovascular studies.

For human disease modeling, the first step is to obtain iPSCs with disease-causing genetic mutations and subsequently differentiate them into the disease-relevant cell types. Disease-specific iPSCs can be obtained through the conversion of somatic cells isolated from patients with genetic mutations. Through the use of this approach, several groups have recently modeled familial dilated cardiomyopathy (5), familial hypertrophic cardiomyopathy (4), Timothy syndrome (6), LEOPARD (lentiginos, electrocardiogram conduction abnormalities, ocular hypertelorism, pulmonary stenosis, abnormal genitalia, retardation of growth, and sensorineural deafness) syndrome (7), arrhythmogenic right ventricular dysplasia (8,9), and long QT syndrome (LQTS) (3,10,11). However, the limitation of this approach is that obtaining patient samples with the desired disease-causing mutation is unpredictable, the subsequent iPSC generation from skin fibroblasts or peripheral blood mononuclear cells is time-consuming, and the disease models generated from various individuals lack an isogenic unaffected control. Most current iPSC studies use age-matched, unaffected cells within the same family pedigree as control (4,10,11), but these are suboptimal because of differences in the genetic background and other confounders.

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Recent advances in genome editing technology allow the direct introduction of specific genetic mutations into the human PSCs to make disease models, with the unedited cells serving as an isogenic control. This technology relies on artificially engineered nucleases to cut and create specific double-stranded breaks at predetermined locations in the genome. The double-stranded breaks can be repaired by the cell's endogenous DNA repair system through either homologous recombination or non-homologous end-joining to produce desired mutations. Several diseases have been modeled with the use of this technique by introducing mutations into the human PSCs (12,13). Although the genome editing technology is powerful for disease modeling, the technology remains a challenge for the non-specialist.

LQTS is an inherited cardiac arrhythmic disease, predisposing the patient to life-threatening ventricular arrhythmias and sudden cardiac death. Mutations in the potassium channels, KCNQ1 (LQTS1) and KCNH2 (LQTS2), account for the 2 most common clinically definite LQTS (14). Because KCNQ1 and

KCNH2 function as tetramers, a substantial number of KCNQ1 and KCNH2 mutants display dominant-negative effect because they interact with wild-type monomer and impair tetramerization (10,11,14). In this study, we successfully modeled LQTS by overexpression of dominant negative mutations in human PSCs, with the use of unedited human PSCs as isogenic controls. We further showed that the LQTS models generated by this approach can be used for drug screening. Our study demonstrates an easy and efficient strategy to generate human disease models.

METHODS

An extended Methods section is available in the [Online Appendix](#).

CELL CULTURE AND MAINTENANCE OF HUMAN PSCs AND PSC-CMs. Human ESCs (WA09, Wicell, Madison, Wisconsin) and iPSCs were cultured on Matrigel-coated plates (ESC qualified, BD Biosciences, San Diego, California) with the use of hESC mTeSR-1 cell culture medium (StemCell Technologies, Vancouver, Canada) under conditions of 37°C, 95% air, and 5% CO₂ in a humidified incubator, as previously described (15). Results for subsequent experiments are based on 1 hESC line (WA09), four unedited iPSC lines (2 from healthy individuals, 1 from a patient with G269S mutation, 1 from a patient with A614V mutation), 4 edited iPSC lines (with mutations R190Q, G269S, and G345E on KCNQ1, and A614V on KCNH2, respectively), and 2 edited ESC lines (with mutations G269S on KCNQ1 and A614V on KCNH2, respectively). All PSC-CMs were maintained in SCT Cardiac Maintenance Media (Stem Cell Theranostics, Menlo Park, California).

VECTOR CONSTRUCTION. The DNA fragment containing EF1a promoter was polymerase chain reaction (PCR)-amplified from the pCDH_EF1_MCS_T2A_copGFP vector (System Biosciences, Mountain View, California) and digested with restriction enzymes MluI and NcoI. The fragment was then inserted into the MluI/NcoI-cut site of the donor plasmid AAV-CAGGS-eGFP backbone (Addgene, Cambridge, Massachusetts) (16). The resultant plasmid was designated AAVS1-EF1a. Human cDNA clones containing KCNQ1 and KCNH2 were obtained from GeneCopoeia (Rockville, Maryland). The coding regions were PCR-amplified and cloned into the AAVS1-EF1a KpnI/AgeI site for the KCNQ1 and AflIII/EcoRV site for the KCNH2. The mutations G569A (R190Q on KCNQ1), G805A (G269S on KCNQ1), G1034A (G345E on KCNQ1), and C821T (A614V on KCNH2) were generated by Mutagenex Inc (Piscataway, New Jersey).

For gene targeting with the use of AAVS1 zinc finger nuclease (ZFNs), refer to the [Online Appendix](#).
For patch-clamp electrophysiology, refer to the [Online Appendix](#).
For imaging of calcium dynamics in genome edited iPSC-CMs, refer to the [Online Appendix](#).

STATISTICAL ANALYSIS. Results are expressed as mean \pm SEM. We used the Student *t*-test for the comparison between 2 normally distributed groups of data. One-way or 2-way analysis of variance followed

by all pairwise multiple comparison procedures, where appropriate, was used for the comparison of multiple groups of data. A value of $p < 0.05$ was considered significant.

RESULTS

ZFN-MEDIATED TARGETED GENE ADDITION INTO AAVS1 SAFE HARBOR LOCUS IN ESCs AND iPSCs. To test the hypothesis that the disease models can be created by overexpression of genes with dominant

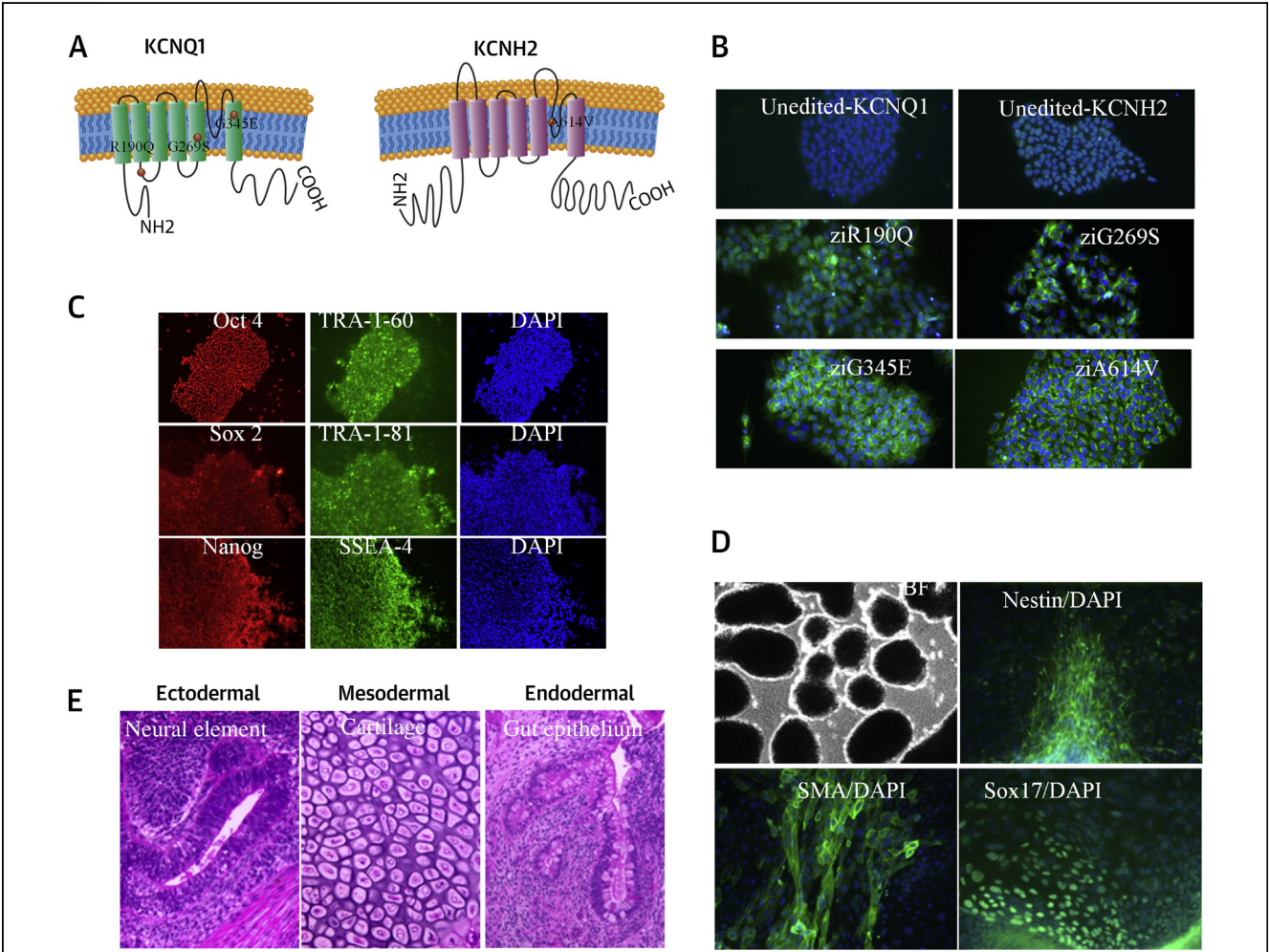


FIGURE 1 Overexpression of the *KCNQ1* and *KCNH2* Genes With Dominant Negative Mutation at the AAVS1 Locus

(A) Schematic representation of the *KCNQ1* and *KCNH2* proteins, with the R190Q mutation located in the cytoplasmic loop between transmembrane segments S2 and S3; G269S mutation located in the S5; G345E mutation located in the S6 of the *KCNQ1* protein; and A614V mutation in the pore region of the *KCNH2* protein. The 6 transmembrane domains (S1 to S6) are flanked by amino (NH2)-terminal and carboxyl (COOH)-terminal regions. **(B)** Transgene expression in the zinc finger nuclease (ZFN)-edited induced pluripotent stem cell (iPSC) lines (ziR190Q, ziG269S, ziG345E, and ziA614V) and control cell lines was confirmed by immunostaining. The upper left unedited iPSCs were stained by use of anti-KCNQ1 antibody; the upper right unedited iPSCs were stained by use of anti-KCNH2 antibody. **(C)** ziG269S expressed pluripotency markers that included Oct4, Tra-1-60, Sox2, Tra-1-81, Nanog, and SSEA4. Blue stainings (right) are 4'-6-diamidino-2-phenylindole (DAPI) staining of nuclei. **(D)** ziG269S formed embryoid bodies (EBs) in vitro as shown by bright-field (BF) (top left) microscopic appearance and by expression of the neuroectoderm marker Nestin (top right), mesoderm marker smooth muscle actin (SMA) (bottom left), and endoderm marker Sox17 (bottom right). **(E)** ziG269S formed teratomas in vivo in immunodeficient mice. The teratomas contain all 3 germ layers, identified here as neural element ectoderm (left), cartilage mesoderm (middle), and epithelium endoderm (right).

TABLE 1 Efficiency of the Gene Addition to the AAVS1 Locus

Cell Line	Donor	PCR-Positive Clones/Total Screened Clones	Clones Screened by Southern Blot	Targeted + Additional Integrations	Correct Targeted Clones	
					Het.	Homo.
iPSC	R190Q	22/23	6	2	3	1
iPSC	G269S	22/23	6	4	2	0
iPSC	G345E	23/23	6	3	1	1
iPSC	A614V	22/23	6	4	0	2
H9	G269S	21/23	6	4	2	0
H9	A614V	21/23	6	3	1	2

Het. = heterozygous; Homo. = homozygous; iPSC = induced pluripotent stem cell; PCR = polymerase chain reaction.

negative mutations, we chose three mutations on KCNQ1 (R190Q, G269S, and G345E) (3,11,17) and 1 mutation on KCNH2 (A614V) (10) to model LQTS1 and LQTS2, respectively (Fig. 1A). To allow a direct comparison of different disease models and avoid mutagenic random integration or epigenetic silencing through viral insertions, the transgenes were inserted into a “safe harbor” AAVS1 locus with the use of zinc finger nuclease (ZFN) technology (Online Fig. S1A) (18). These ion gene mutants were subcloned into the donor vector driven by the EF1a promoter flanked by short (800 bp) stretches of homology to the ZFN target site on chr 19 in exon 1 of the *PPP1R12C* gene (16). The donor construct and the ZFN expression vector were introduced into iPSCs by electroporation. The ZFN-edited iPSC lines with mutations R190Q, G269S, G345E, and A614V are designated as ziR190Q, ziG269S, ziG345E, and ziA614V, respectively.

In addition to these four ZFN-edited iPSC lines, we also introduced G269S-KCNQ1 and A614V-KCNH2 mutants into human ESCs (WA09); these lines are designated as H9-G269S and H9-A614V, respectively.

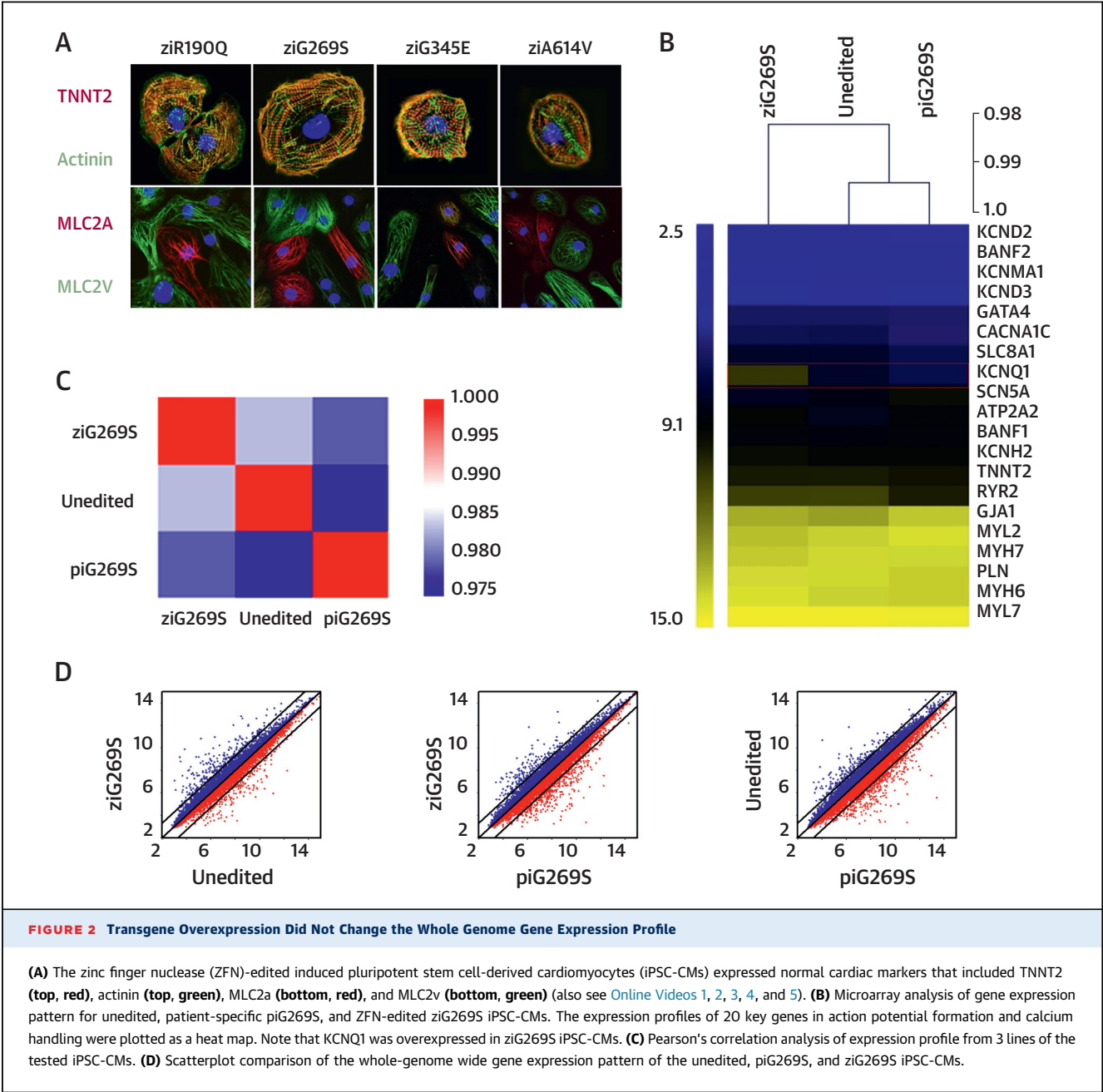
After puromycin selection, 23 clones for each construct were screened by genomic PCR, 95.2% (197/207) of which carried the transgenic cassette at the ZFN-specified location (Online Fig. S1B, Table 1). Of these PCR-positive clones, 6 clones for each mutation were further screened by Southern blotting (Online Fig. S1C, Table 1). The results revealed that all the clones contained 1 or 2 copies of targeted gene addition, except for 1 from ziG345E. We next genotyped a panel of putative ZFN off-target sites for each mutation (Online Fig. S2), and data demonstrate all to be wild-type, in agreement with previous studies on the robust specificity of this ZFN set (16,18). After extensive genetic characterization, immunostaining confirmed the transgene expression (Fig. 1B, Online Fig. S3).

GENOME EDITING DOES NOT AFFECT PLURIPOTENT STEM CELL CHARACTERISTICS. To investigate

whether the genetic engineering and transgene overexpression would affect stemness, we tested the pluripotency of our cells after ZFN editing and transgene overexpression and observed that all the transgenic cell lines displayed normal morphology relative to control unmodified iPSCs (Online Fig. S4). Additional testing revealed that transgenic cells maintained their pluripotent state, as indicated by the expression of pluripotency markers, such as Oct4, Tra-1-60, Sox2, Tra-1-81, Nanog, and SSEA4 (Fig. 1C, Online Fig. S5). Functionally, these cells were capable of differentiation into all three germ layers both in vitro (Fig. 1D, Online Fig. S6A) and in vivo (Fig. 1E, Online Fig. S6B). The transgenic cells exhibited normal karyotypes (Online Fig. S7). Taken together, these results show that transgenic iPSCs maintained their pluripotent potential after genetic engineering and transgene overexpression.

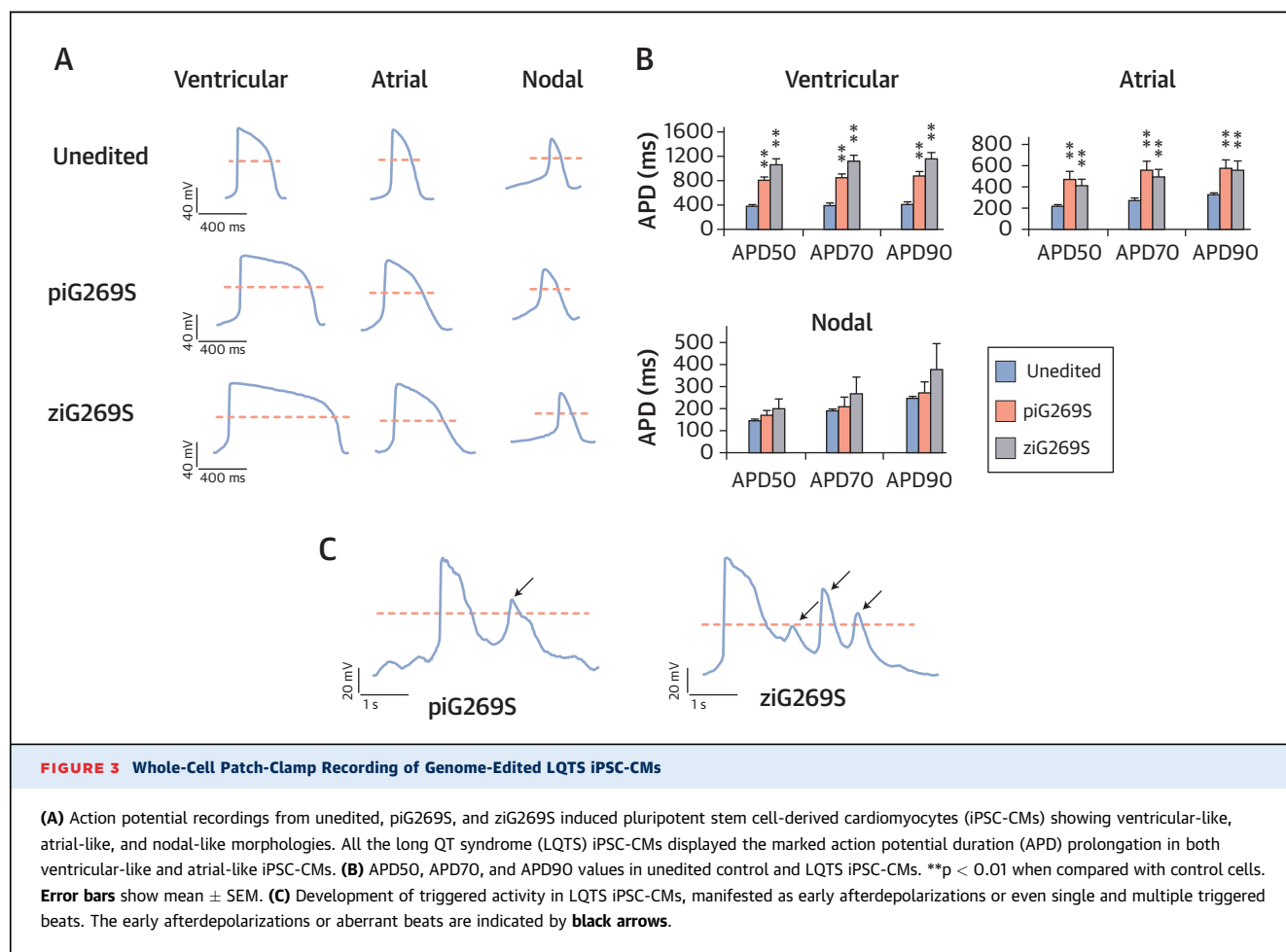
ZFN EDITING AND TRANSGENE EXPRESSION DOES NOT INFLUENCE WHOLE GENOME EXPRESSION PROFILE.

We next differentiated the transgenic iPSCs into cardiomyocytes (iPSC-CMs) in vitro through the use of a modified monolayer protocol, as previously described (19). After 12 days of differentiation, cell beating was observed for both unedited (Online Video 1) and transgenic (Online Video 2 for ziR190Q, Online Video 3 for ziG269S, Online Video 4 for ziG345E, and Online Video 5 for ziA614V) iPSC-CMs. The cardiac marker expression (α -actinin, TNNT2, MLC2a, and MLC2v) in the transgenic iPSCs was similar to those seen in control unedited iPSCs (Fig. 2A). To study whether the genetic engineering and ion channel gene overexpression influence the expression of other genes in iPSC-CMs, gene expression array (Affymetrix, Santa Clara, California) was performed with genome-edited ziG269S iPSC-CMs, patient-derived G269S iPSC-CMs (piG269S) (3), and unedited iPSC-CMs. As shown in Figure 2B, 20 genes that were closely related to the action potential forming, calcium handling, and conductivity of the iPSC-CMs were compared among the three groups. As expected, the KCNQ1 gene is upregulated in the ziG269S group, yet no obvious changes were shown in the expression level of other genes. Overall, our microarray analysis showed very similar gene expression patterns in these 3 groups, and the correlation between any 2 groups are no less than 97.5% (Figs. 2C and 2D). In addition, we also tested the expression of a panel of ion channel gene expression by quantitative PCR, and their expression was similar to that for the unedited iPSC-CMs, except the transgenes were overexpressed (Online Fig. S8).



TRANSGENIC iPSC LINES RECAPITULATED LQTS PHENOTYPES. To assess whether the genome-edited iPSC-CMs recapitulated the LQTS phenotype, patch-clamp recordings were performed from single cells, and cardiac action potentials were obtained. We first recorded the action potentials in parallel through the use of iPSC-CMs derived from unedited iPSC, ziG269S, and piG269S lines. Consistent with previous reports, cardiac action potentials were recorded from single iPSC-CMs, and all three subtypes of myocytes (e.g., ventricular-like, atrial-like, and nodal-like)

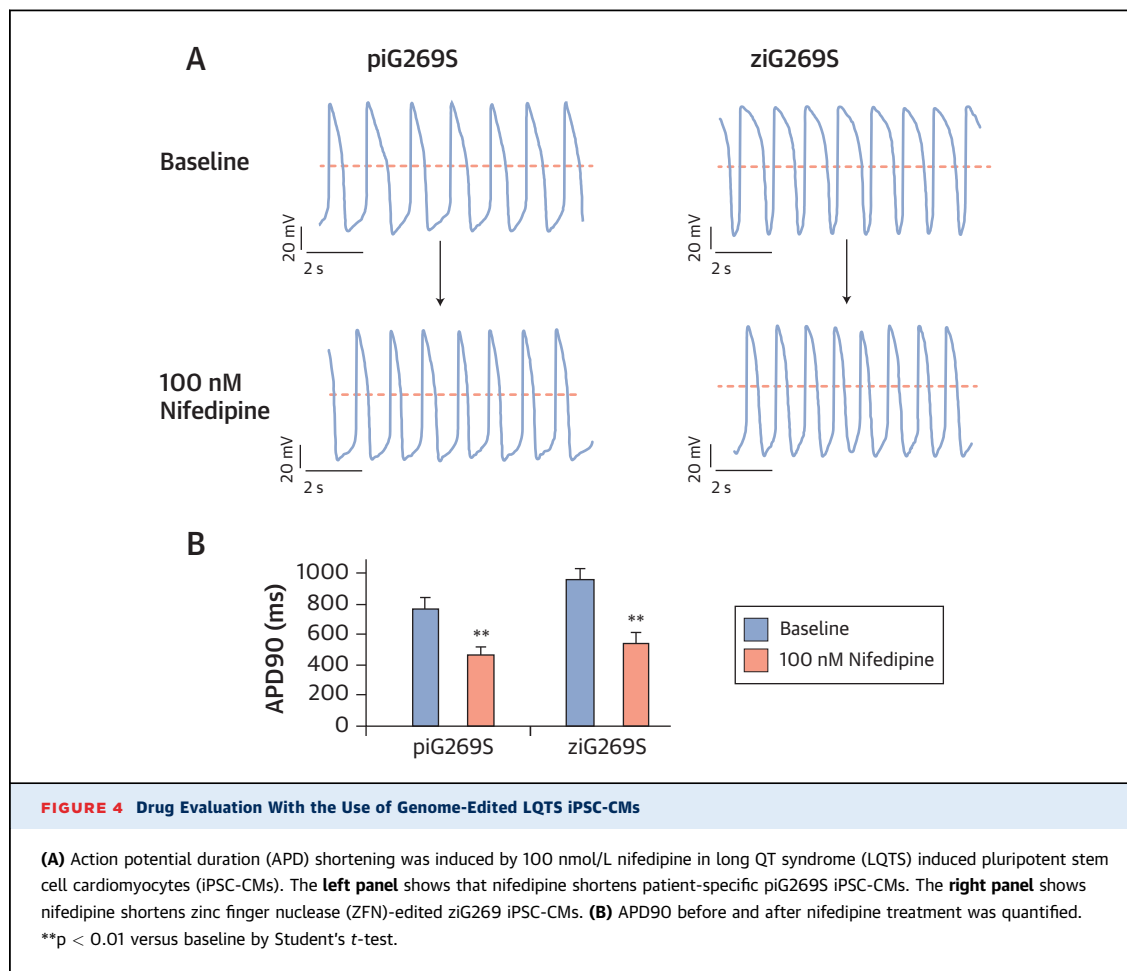
were detected in the three groups (Figs. 3A and 3B). Key parameters of action potentials were collected and assessed (Online Table S1). There were no significant changes observed in key parameters except action potential duration at 90% repolarization (APD90). The ventricular-like and atrial-like myocytes derived from ziG269S exhibited significantly longer APD compared with unedited iPSC-CMs, which are similar to the data obtained from piG269S iPSC-CMs. More interestingly, both the ZFN-edited ziG269S iPSC-CMs and patient-specific piG269S iPSC-CMs



displayed early afterdepolarizations, which were not present in unedited iPSC-CMs (Fig. 3C). In addition, we also recorded action potentials from ziR190Q, ziG345E, ziA614V, and patient-derived A614V (piA614V) iPSC-CMs (10), all of which showed significantly prolonged APDs compared with the unedited iPSC-CMs (Online Fig. S9, Online Table S1), consistent with previous studies (10,11). In conclusion, ZFN-edited LQTS iPSC lines can faithfully recapitulate the disease phenotype, which is similar to the one obtained from the patient-specific, mutation-matched iPSC lines.

GENOME-EDITED iPSC-CMs AS A NEW PLATFORM FOR DRUG TESTING. To evaluate the suitability of the ZFN-edited iPSC disease model as a tool for drug screening, we next tested nifedipine (a specific Ca^{2+} blocker) on both ziG269S and piG269S iPSC-CMs. Consistent with previous studies (10), when tested on piG269S iPSC-CMs with a long APD baseline, nifedipine (100 nmol/l) can shorten the APD as the result of its Ca^{2+} blockade effect (Figs. 4A and 4B). Interestingly, nifedipine also can significantly shorten the APD when tested in ziG269S iPSC-CMs (Figs. 4A and 4B),

indicating that the genome-edited iPSC-CMs can be used as a new platform for drug testing. To confirm the conclusion above, we recorded spontaneous calcium transient in iPSC-CMs (Online Table S2). Compared with unedited control iPSC-CMs, both ziG269S and piG269S iPSC-CMs exhibited greater prolongation of transient duration 90 by $63.6 \pm 7.9\%$ and $60.8 \pm 8.6\%$, respectively (Online Table S2). After treatment with nifedipine, the transient duration 90 in both ziG269S and piG269S iPSC-CMs was significantly shortened, similar to the results obtained from patch clamping recording. We further tested the effect of pinacidil, a KATP channel opener that can augment outward potassium currents. Interestingly, pinacidil (100 nmol/l) also can shorten the transient duration 90 of both ziG269S and piG269S iPSC-CMs (Online Fig. S10, Online Table S2). Taken together, these data suggest that both genome-edited iPSC models and patient-derived iPSC models can be used for drug screening, with the added advantage that the former approach obviates patient recruitment, which can be time-consuming and unpredictable (Central Illustration).



DISCUSSION

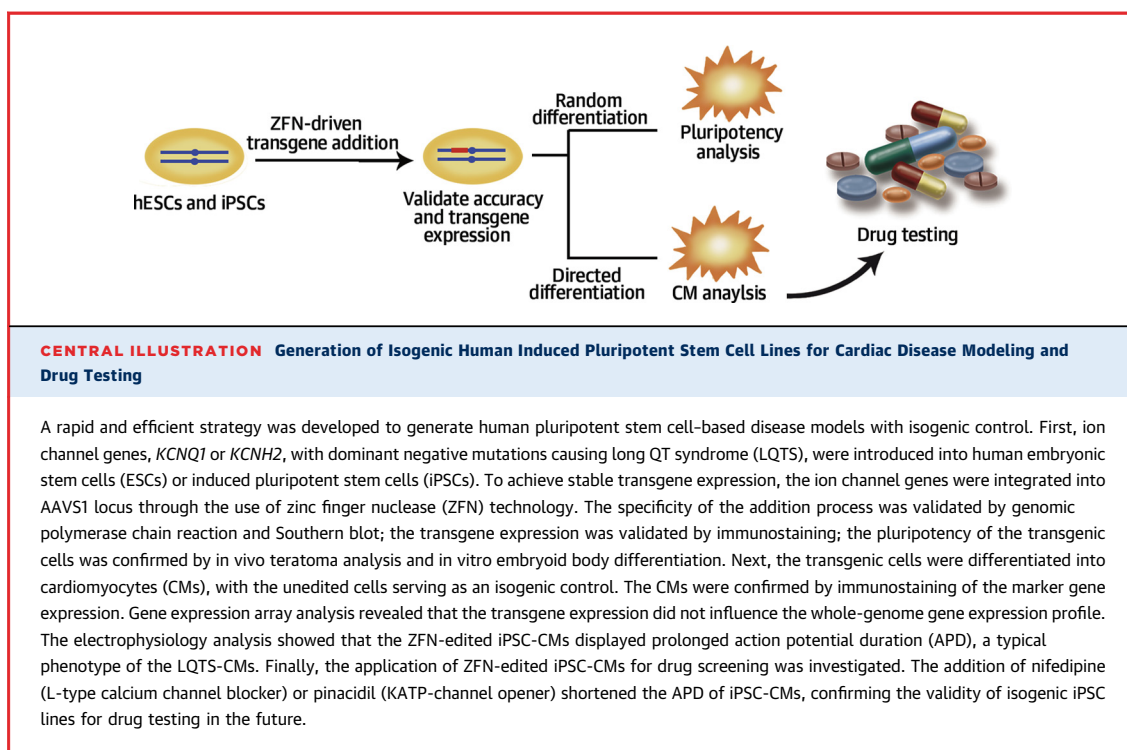
In the present work, we demonstrated a new strategy to create human PSC-based disease modeling by overexpression of genes with dominant negative mutations. This strategy is easy and highly efficient. With the use of this strategy, we successfully generated six cell lines representing LQTS1 and LQTS2 in a short period of time. Importantly, the unedited parent cell lines can serve as an isogenic control. We further showed that this strategy can be used as an effective platform for drug screening ([Central Illustration](#)).

Human PSCs represent an excellent system for disease modeling because they can be potentially differentiated to any cell types present in the human body. However, obtaining human PSCs with specific disease-causing genetic mutations can be unpredictable and time-consuming. By contrast, genetic engineering strategy to generate iPSC-based disease models is much easier and faster. The reagents and methods to engineer the AAVS1 locus already exist and have been optimized ([16,18](#)). An investigator with

basic molecular biology skills can generate appropriate cell lines in three steps: identifying a gene with desired dominant negative mutations, cloning the gene into the targeting vector, and integrate the construct into the AAVS1 locus, following the provided protocol. One person can generate several disease models in 3 months with the use of this strategy.

The quality of the studies depends on the availability of appropriate controls, and hence, any phenotypes observed in the PSC-derived cells should only be interpreted through comparison with control cells, which ideally would have the same genetic background as the mutant PSCs and would differ only in the disease-linked gene. The usual practice is to generate control iPSC lines from a close relative of the patient who is age-matched and unaffected by the disease within the same pedigree, but they are often unavailable and represent only an approximate control.

Differences in genetic background are another major concern. Even in studies in which healthy siblings have been used as controls for disease patients, only ~50% of the genome is shared between any



siblings. Furthermore, phenotypic differences could be the result of DNA variants in the other ~50% of the genome rather than the disease-associated mutations (20). By contrast, the strategy we developed in this study can use un-modified parental cell line as an isogenic control, which genetically differs from the transgenic cell lines only in the introduced gene.

In our study, the ion channel genes with dominant negative mutations were overexpressed in the transgenic cells. The iPSC-CMs derived from transgenic PSCs showed normal cardiac gene expression, without changing the whole genome gene expression profile of the resultant cells. These iPSC-CMs recapitulated LQTS phenotypes and had prolonged APD. Importantly, the iPSC-CMs derived from both patient-specific and genome-edited iPSCs showed similar response to drugs that affect ion channels, demonstrating that transgenic iPSCs can be used for drug screening in the future.

It is widely recognized that human PSC-CMs are immature. Previous studies have shown that human ESC-CMs expressed cardiac genes at levels similar to those found in 20-week fetal heart cells (21,22). Mummery et al. (23) have shown that the upstroke velocities for the ventricular-like cells were low but comparable to those in cultured human fetal ventricular cardiomyocytes. Nevertheless, several publications have revealed that these cells are capable of capturing specific traits of an electrical disease of the

heart, including LQTS1 (3,11), LQTS2 (10), LQTS3 (24), and LQTS8 (6,25).

Finally, disease-specific iPSCs have enormous potential for drug screening (26). Proof of principle for iPSC models as a platform for small-molecule and chemical testing already exists. For example, neurons from spinal muscular atrophy (27) and familial dysautonomia (28) displayed in vitro defects that could be corrected by candidate drugs, and the effect of β -blocking drugs has been evaluated for LQTS iPSC-CMs (11). However, the possibility that the genetically edited PSCs could be used as a platform for the drug screening was not investigated until now.

In this study, we demonstrate that the LQTS phenotype of the genome edited iPSCs-CMs can be corrected by the nifedipine and pinacidil. We further showed that the genetically edited LQTS models worked equally well on the patient LQTS iPSC models. In summary, our study provides investigators a robust and reliable system to generate isogenic iPSC lines, which will be useful for disease modeling, drug discovery, and regenerative medicine in the future (26).

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PERSPECTIVES

COMPETENCY IN MEDICAL KNOWLEDGE: Introducing ion channel genes with dominant negative mutations causing long QT syndrome into human induced pluripotent stem cells can generate cardiomyocytes that display the long QT phenotype, including prolonged action potential duration.

TRANSLATIONAL OUTLOOK 1: The integration of transgenes into specific loci through the use of zinc finger nuclease technology avoids random mutagenic

integration and epigenetic silencing through viral insertions and may be applicable to modeling of other diseases caused by dominant negative mutations.

TRANSLATIONAL OUTLOOK 2: This strategy has the potential to accelerate modeling of genetic diseases for identification of novel drug targets and testing pharmacological strategies in vitro to improve the likelihood of clinical efficacy.

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KEY WORDS disease models, drug testing, genome editing, long QT syndrome, stem cells

APPENDIX For supplemental tables, figures, and videos, please see the online version of this paper.